References
Cathou, R. E., & Dorrington, K. J. (1975) in *Biological Macromolecules, Subunits in Biological Systems* (Fasman, G. D., & Timasheff, S. N., Eds.) Part C, Vol. 7, p. 91, Marcel Dekker, New York, N.Y.
Colman, P. M., Deisenhofer, J., Huber, R., & Palm, W. (1976) *J. Mol. Biol. 100,* 257.
Crick, F. H. C., & Magdoff, B. (1956) *Acta Crystallogr. 9,* 901.
Edmundson, A. B., Schiffer, M., Wood, M. K., Hardman, K. D., Ely, K. R., & Ainsworth, C. F. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36,* 427.
Edmundson, A. B., Schiffer, M., Ely, K. R., & Wood, M. K. (1972) *Biochemistry 11,* 1822.
Ely, K. R., Girling, R. L., Schiffer, M., Cunningham, D. E., & Edmundson, A. B. (1973) *Biochemistry 12,* 4233.
Fett, J. W., Deutsch, H. F., & Smithies, O. (1973) *Immunochemistry 10,* 115.
Huber, R., Deisenhofer, J., Colman, P. M., Matsushima, M., & Palm, W. (1976) *Nature (London) 264,* 415.
Labaw, L. W., & Davies, D. R. (1971) *J. Biol. Chem. 247,* 3760.
Laemmli, U. K. (1970) *Nature (London) 222,* 680.
Laschinger, C. A. (1976) M.Sc. Thesis, University of Toronto.
Lopes, A. D., & Steiner, L. A. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 1003.
Low, B. W., & Richards, F. M. (1952) *J. Am. Chem. Soc. 74,* 1660.
Matthews, B. W. (1968) *J. Mol. Biol. 33,* 491.
Michaelsen, T. E., Frangione, B., & Franklin, E. C. (1977) *J. Biol. Chem. 252,* 883.
Milstein, C., & Pink, J. R. L. (1970) *Prog. Biophys. Mol. Biol. 21,* 209.
Noelken, M. E., Nelson, C. A., Buckley, C. E., III, & Tanford, C. (1965) *J. Biol. Chem. 240,* 218.
Sarma, V. R., Silverton, E. W., Davies, D. R., & Terry, W. D. (1971) *J. Biol. Chem. 247,* 3753.
Schiffer, M., Girling, R. L., Ely, K. R., & Edmundson, A. B. (1973) *Biochemistry 12,* 4620.
Silverton, E. W., Navia, M. A., & Davies, D. R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Zeppezauer, M., Eklund, H., & Zeppezauer, E. S. (1968) *Arch. Biochem. Biophys. 126,* 564.

Characterization and Affinity Labeling of Nucleotide Binding Sites of Bacterial Plasma Membrane Adenosine Triphosphatase (F_1)†

Franz W. Hulla,* Michael Höckel, Michael Rack,* Sergio Risi, and Klaus Dose

ABSTRACT: 6-[(3-Carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate (Nbs^{6}ITP), a chemically reactive ATP analogue, has been used to label nucleotide binding sites of the plasma membrane ATPase (F_1) from *Micrococcus* species ATCC 398. Nbs^{6}ITP is not hydrolyzed by the ATPase. Binding of the ATP analogue to the enzyme slowly (within hours) produces an irreversible inhibition of the ATPase which shows a pseudo-first-order kinetics. This "long-term" inhibition is preceded by a reversible "short-term" competitive inhibition. The K_m value for the "long-term" inhibition and the K_i value for the competitive inhibition are identical, indicating that the binding of Nbs^{6}ITP occurs at the same site(s) in both cases. Nbs^{6}ITP has been labeled with ^{32}P in the β and γ positions to elucidate the stoichiometry of nucleotide binding. The degree of inhibition of the enzyme is stoichiometrically related to the number of nucleotides bound. One-hundred percent inhibition is correlated by extrapolation with the incorporation into the F_1 complex of about six nucleotides which are not released by gel filtration in glycerol containing buffer. These "firmly" bound nucleotides are to some extent covalently bound to the enzyme as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. At 80% inhibition 1.7 of a total of 4.8 bound nucleotides are covalently bound. Covalent binding of Nbs^{6}ITP to F_1 occurs exclusively at the β subunit(s) of the enzyme. A cysteinyl residue is not involved in this reaction.

Membrane-bound ATPases[1] from mitochondria, chloroplasts, and bacteria are enzymes of very complex structure and function. The F_1 part of this protein complex can be solubilized in dilute buffers. It consists of up to five different subunits depending on the conditions of the preparation. The F_0 part of the ATPase complex is a hydrophobic integral membrane protein, which can only be solubilized in the presence of detergents. If bound to intact vesicular membranes and linked to energy-producing electron transport reactions, the ATPase is able to synthesize ATP from ADP and P_i. Conversely, the energy of ATP hydrolysis can be used for promotion of energy-dependent reactions such as active transport (Tsuchiya & Rosen, 1975).

Because the site of ATP synthesis or hydrolysis is the F_1 factor, the nucleotide binding sites of F_1 are of central interest for the elucidation of the complex enzyme mechanism. There

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[1] Abbreviations used: F_1 ATPase, adenosinetriphosphatase (EC 3.6.1.3); Dnps^{6}ITP, 6-[(2,4-dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate; Nbs^{6}ITP, 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate; P_i, inorganic phosphate; Nbf-Cl, 4-chloro-7-nitrobenzofurazan.

0006-2960/78/0417-0823$01.00/0

have been numerous studies of the number and function of the nucleotide binding sites of the F_1 factor (Garrett & Penefsky, 1975; Abrams et al., 1975; Cantley & Hammes, 1975; Maeda et al., 1976; Lee et al., 1977).

Different nucleotide binding sites are proposed to be involved in ATP hydrolysis, oxidative phosphorylation, and enzyme regulation (Pedersen, 1975; Schuster et al., 1975). There may be multiple binding sites of the same function, because the subunit stoichiometry suggests the presence of two or three catalytic units on F_1 (Höckel et al., 1978). Moreover, variability of nucleotide binding sites during the enzymic reaction cycle, e.g., transitions from "loose" to "tight" sites and vice versa, is indicated by a number of experiments (Boyer, 1977). Such transitions could also express functional interconversions of nucleotide binding sites at different states of the enzyme action.

Thus, the pattern of nucleotide binding sites on F_1 appears to be extremely complex. In the present study we report the results of affinity labeling experiments with the ATP analogues Dnps^{6}ITP and Nbs^{6}ITP and purified F_1 preparations of *Micrococcus* sp. plasma membranes.

Materials and Methods

General. All chemicals were of analytical grade, purchased from Merck (Darmstadt, GFR) or Serva (Heidelberg, GFR); otherwise specifications are given. Dried organic solvents were stored over a molecular sieve (0.4 nm). [^{32}P]Pyrophosphate was from NEN (Boston, Mass.).

Analytical Disc Gel Electrophoresis. Analytical disc gel electrophoresis in Tris/glycine buffer has been carried out on 5% polyacrylamide gels as described earlier (Hulla et al., 1976). Sodium dodecyl sulfate disc gel electrophoresis was performed on 7.5% polyacrylamide gels according to Weber & Osborn (1969). Disulfide cleaving reagents were omitted, which had no effect on the polypeptide pattern on the gel (Risi et al., 1978).

Preparation of F_1 ATPase. The enzyme was prepared according to a modified procedure described by Risi et al. (1977). This procedure involves a lysozyme and DNase treatment of the cells followed by an osmotic shock with solubilization of the F_1 ATPase. Purification to homogeneity includes $(NH_4)_2SO_4$ precipitation, chromatography on Bio-Gel A-1.5m and A-0.5m (Bio-Rad, München, GFR), and finally on DEAE-Sepharose CL-6B (Pharmacia, Freiburg, GFR) in the presence of diisopropyl fluorophosphate (Serva, Heidelberg, GFR).

ATPase Assay. ATPase activities were measured in 0.1 M Tris/HCl buffer (pH 8.0) in the presence of 0.5 mM ATP and 5 mM $CaCl_2$ at 37 °C by determining the liberated orthophosphate. One unit (U) is defined as the number of micromoles of inorganic phosphate liberated during 1 min at 37 °C. The inorganic phosphate was sampled according to the method of Fiske & Subbarow (1925) in a continuous flow apparatus.

Protein Determination. Protein concentrations were determined according to Lowry et al. (1951) with bovine serum albumin (Boehringer, Mannheim, GFR) as standard. For conversion into ATPase dry weight the concentration values were multiplied by 0.7. Alternatively, protein determinations were carried out spectrophotometrically using an absorption coefficient of $A^{1\%}_{280} = 6.0$.

Determination of Radioactivity. The ^{32}P and ^{14}C radioactivity was determined with a Packard Tri-Carb liquid scintillation counter, Model 3380. Quenching corrections were made when necessary. Glycerol containing samples were counted in plastic vials in the presence of 0.6 mL of H_2O and 10 mL of scintillator Unisolve 100 (Koch-Light, Colnbrook Bucks, England). Gel slices were measured in glass vials containing 1 mL of solubilizer TS 1 (Koch-Light), 1 mL of 1 N HCl, and 10 mL of Unisolve 100.

Synthesis of Dnps^{6}ITP. The ATP analogue 6-[(2,4-dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate (Dnps^{6}ITP) was synthesized according to Fasold et al. (1977).

6-Mercaptopurine ribonucleoside (6-thioinosine) (Waldhof Chemie, Mannheim, GFR) was isopropylidated at the 2′ and 3′ positions of the riboside according to Clayton et al. (1968). The isopropylidated thioinosine was dried and stored over P_2O_5 in vacuo. The introduction of the reactive sulfur bond in the 6 position was carried out according to Faust et al. (1974).

The product, 6-[(2,4-dinitrophenyl)thio]-9-β-D-(2′,3′-*O*-isopropylidene)ribofuranosylpurine, was dried and stored over P_2O_5 in vacuo. Purity was checked by thin-layer chromatography in acetic acid–butyl acetate on silica gel (Riedel de Haen, Hannover, GFR) (R_f 0.3). Spraying with β-mercaptoethanol solution at pH 8.0 liberates the intensively yellow-colored dinitrothiophenolate. The reaction may be followed at 418 nm in an ultraviolet spectrometer.

The synthesis of 6-[(2,4-dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-monophosphate was carried out according to the method of Yoshikawa et al. (1967). Yields were improved by dissolving the precipitate in dilute HCl and by repeating the whole process beginning with readjusting the pH to 7.0 by addition of triethylamine. The resulting barium salt of the 6-[(2,4-dinitrophenyl)thio]-9-β-D-(2′,3′-*O*-isopropylidene)ribofuranosylpurine 5′-monophosphate was dried by lyophilization, and stored like 6-[(2,4-dinitrophenyl)thio]-9-β-D-(2′,3′-*O*-isopropylidene)ribofuranosylpurine. Purity was checked on PEI-cellulose in a system as described below for the ATP analogue.

For the synthesis of the triphosphate the barium salt of the 6-[(2,4-dinitrophenyl)thio]-9-β-D-(2′,3′-*O*-isopropylidene)-ribofuranosylpurine 5′-monophosphate dissolved in water was converted into the free acid by passing it through a Dowex WX 50 column in the H^+ form; simultaneously the isopropylidene group was removed. The free acid of the monophosphate was lyophilized and used immediately for the triphosphate synthesis carried out essentially according to the procedure of Michelson (1964). All solvents used at this step were dried carefully to obtain appreciable yields.

Tributylammonium phosphate was prepared as described by Moffat (1964). The resulting lithium salt of the triphosphate was dissolved in water and lyophilized; it can be stored over P_2O_5 in vacuo for extended periods of time.

Purity was checked on PEI-cellulose thin-layer chromatography sheets (Merck, Darmstadt, GFR), in a system consisting of 2 N formic acid and 0.5 M LiCl. The ATP analogue migrates more slowly than ADP, and about as fast as ATP, with an R_f of 0.3. 6-[(2,4-Dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-monophosphate has an R_f of 0.85; it migrates as fast as AMP. The spots can be detected on the chromatograms by ultraviolet absorption or by spraying with β-mercaptoethanol solution, buffered at pH 8.0, as described for 6-[(2,4-dinitrophenyl)thio]-9-β-D-(2′,3′-*O*-isopropylidene)ribofuranosylpurine and 6-[(2,4-dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-monophosphate. The ultraviolet absorbance spectrum of the compounds dissolved in 0.1 M Tris/HCl (pH 7.0) showed a maximum at 275 nm. Thioinosine (10 g) yields approximately 800 mg of Dnps^{6}ITP.

Synthesis of Nbs^{6}ITP and [^{32}P]Nbs^{6}ITP. This ATP analogue was synthesized from thioinosine, which was first isopropylidated according to Hampton & Maguire (1961). The

nucleoside was then converted into 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-(2′,3′-*D*-isopropylidene)ribofuranosylpurine by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and the resulting nucleoside thioether was phosphorylated at the 5′ group of the riboside moiety with $POCl_3$; for details see Hulla & Fasold (1972). The monophosphate, 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-monophosphate, was activated with diphenyl phosphochloridate, which yields the triphosphate, Nbs^{6}ITP, by addition of bis(tri-*n*-butylammonium) pyrophosphate. The purification process was carried out as described earlier (Hulla et al., 1976), with the following modification: The final concentrate (50 mL) was applied to a DEAE-Sephadex A-25 column (1.5 × 30 cm) in the chloride form, and elution was achieved with a linear gradient of water–0.8 M LiCl (1500 mL each; pH 7.0) instead of a triethylammonium bicarbonate gradient. The collected fractions containing the ATP analogue were lyophilized, and the resulting residue was dissolved by a minimum amount of water adjusted to pH 6.5 with LiOH. Nbs^{6}ITP was then precipitated with ethanol–acetone (1:4) (Michelson, 1964) and the collected precipitate was washed twice with ethanol. Nbs^{6}ITP can be stored in a brown flask in vacuo over P_2O_5 for several months without decay. The yield was 55% for the final step.

The synthesis of the ^{32}P-labeled nucleoside triphosphate was performed very similarly to that of the nonradioactive ATP derivative. The bis(tri-*n*-butylammonium) [^{32}P]pyrophosphate, however, was added prior to the diphenyl phosphochloridate activated monophosphate. After incubation for 45 min the nonradioactive bis(tri-*n*-butylammonium)pyrophosphate was added and allowed to react for 1.5 h to complete the reaction. The specific radioactivity of [^{32}P]Nbs^{6}ITP was 35 mCi/mmol. ^{32}P was incorporated in the β and γ positions of the triphosphate.

"Short-Term" (Reversible) Inhibition Kinetics. The experiments were carried out in 4 mL of 100 mM Tris/HCl buffer (pH 8.0), containing 1 mM $MgCl_2$, 2.25 μg of F_1 ATPase, as well as the substrate and inhibitor (Nbs^{6}ITP) concentrations as shown in the corresponding figure.

The enzyme was first incubated in Tris buffer for 1 min, then the inhibitor was added, and after stirring for 1 min the enzymic reaction was started by addition of the substrate and followed spectrophotometrically.

"Long-Term" (Irreversible) Inactivation and Affinity Labeling. The samples contained in a volume of 2 mL: 50 mM Tris/HCl buffer (pH 8.0), 1 mM $MgCl_2$, 0.76 mg of F_1, and 4.5×10^{-5} M Nbs^{6}ITP. They were incubated for several hours at 35 °C in the dark. The amounts of nucleotides bound to F_1 were assayed as follows. Aliquots were withdrawn at distinct intervals and saturated with $(NH_4)_2SO_4$; the precipitated protein was resolubilized after centrifugation in 400 μL of "glycerol buffer", containing 50 mM Tris/HCl (pH 8.0), 4 mM EDTA, and 50% glycerol. After reincubating the protein solution for 1 h at 35 °C, it was chromatographed on a Sephadex G-50 column (Pharmacia, Freiburg, GFR) in "glycerol buffer". Column dimensions were 0.9 × 30 cm, flow rate was 2 mL/h, and fraction volume was 0.8 mL.

The two fractions containing the bulk of the protein were combined and the protein content, enzymic activity, and ^{32}P radioactivity were determined. The measured radioactivity represents the amount of Nbs^{6}ITP "firmly" plus covalently bound to the enzyme. In order to find out the number and localization of the covalently bound ITP on the F_1 molecule, 150-μL aliquots of the solution containing about 20 μg of protein were subjected to sodium dodecyl sulfate disc electrophoresis. To determine the amount of covalently bound radioactivity the unstained gel was cut into 2-mm slices immediately after electrophoresis. Each slice was kept in a glass vial with 1 mL of solubilizer TS 1 for 24 h at 50 °C before counting.

The distribution of covalently attached radioactivity between the ATPase subunits has been assayed by the following procedures. (1) The gel was split after electrophoresis, one part was cut into unstained slices, the other was stained for the protein bands. (2) The whole gel was stained and destained and subsequently cut into slices. Further treatment of the gel slices was carried out as described above.

Distribution of Sulfhydryl Groups. The samples containing 0.1 M phosphate buffer (pH 7.2), 1% sodium dodecyl sulfate, 2 mM EDTA, and 1 mg of F_1 in a volume of 0.5 mL were incubated under nitrogen at 37 °C for 2 h. Subsequently 0.1 mL of 4 mM 5,5′-[^{14}C]dithiobis(2-nitrobenzoic acid) (19.2 mCi/mmol, CEA, Gif-sur-Yvette, France) in 0.1 M phosphate buffer (pH 7.2) was added. After 15 min the excess 5,5′-dithiobis(2-nitrobenzoic acid) was removed by gel filtration on Sephadex G-25 medium (Pharmacia, Freiburg, GFR) in 0.1 M phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate and 2 mM EDTA. The column size was 0.9 × 30 cm, the flow rate was 4 mL/h, and the fraction volume was 0.8 mL. The two fractions containing the bulk of the protein were then subjected to sodium dodecyl sulfate disc electrophoresis. Each gel was loaded with an aliquot of 0.1 mL (20–30 μg of protein). The association of the ^{14}C radioactivity with the corresponding enzyme subunit was determined as described above.

Molecular Weight Determination. The molecular weight of F_1 was determined applying the sedimentation-equilibrium method. A Beckman Spinco Model E ultracentrifuge with a Yphantis cell was used. Experiments were performed with three different enzyme concentrations at 6000 rpm. A partial specific volume of 0.726 cm^3/g calculated from the amino acid analysis was taken for evaluation.

Results

The affinity labels Nbs^{6}ITP and Dnps^{6}ITP are substrate analogues, which are suited for selective modification of amino acid side chains (Fasold et al., 1977). The reactive group of both analogues is a sulfide bond activated by a nitrophenyl group and the quasiaromatic purine ring system. The affinity labels react preferentially with aliphatic mercapto groups. Dnps^{6}ITP from pH 7.5 upward and Nbs^{6}ITP from pH 8.0 upward yield a new and more stable sulfide bond between the SH group of cysteine and the C-6 position of the nucleotide moiety, while the nitrothiophenolate is liberated. This type of an ATP analogue therefore provides a reagent for affinity labeling of proteins carrying SH groups near or at the nucleotide binding site. However, because of reactivity changes of functional groups in the microenvironment of a binding site of a protein molecule, other functional groups, too, e.g., phenolic OH groups of tyrosine, might be able to react with the C-6 of the purine ring via a tetrahedral intermediate.

In the beginning of our experiments Dnps^{6}ITP was preferred, because it reacts under more physiological conditions (pH 7.5) with amino acid side chains than Nbs^{6}ITP. However, Dnps^{6}ITP, entirely free from inorganic phosphate, is difficult to synthesize. The presence of inorganic phosphate can hamper the quantitative evaluation of the incorporation rate into the protein, if ^{32}P-labeled nucleoside triphosphates are used. Therefore, the final kinetic treatment of the F_1 ATPase and the locating of the ATP binding sites have been carried out with Nbs^{6}ITP, which can be obtained free from inorganic phosphate more easily.

Incubations of Nbs^{6}ITP with F_1 in the presence of Mg^{2+}

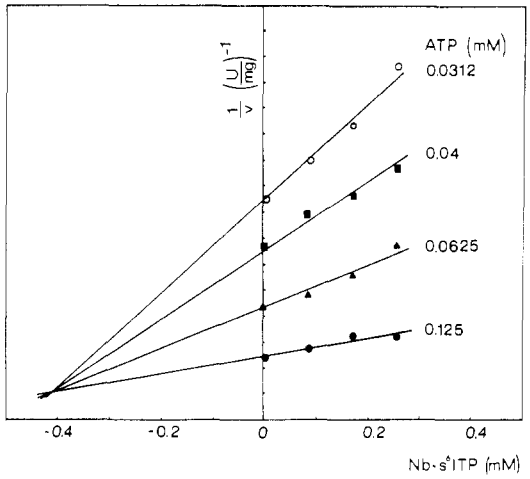

FIGURE 1: Graphical illustration of the competitive inhibition of the F_1 ATPase by Nbs^{6}ITP and determination of the K_i value (method of Dixon).

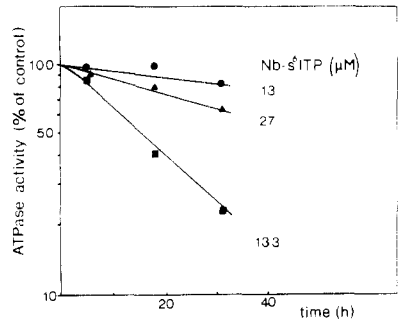

FIGURE 2: "Long-term" irreversible inactivation of F_1 ATPase by Nbs^{6}ITP according to a pseudo-first-order kinetics. Nbs^{6}ITP concentrations are indicated in the figure.

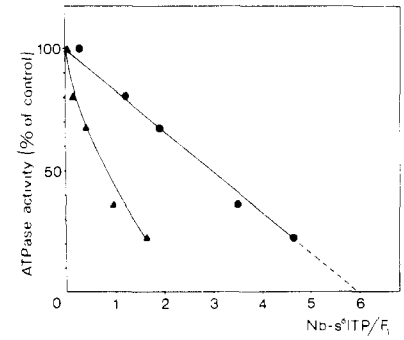

FIGURE 3: Linear relationship between the residual ATPase activity and the number of Nbs^{6}ITP nucleotides incorporated into the ATPase: covalently attached nucleotides (▲); "firmly" bound plus covalently attached nucleotides (●)

ions at pH 8.0, even for extended periods, revealed that this ATP analogue is not accepted as a substrate by the ATPase, i.e., no enzymic liberation of inorganic phosphate occurs. Yet, short-period and long-period incubations showed defined interactions between the enzyme and the nucleotide analogue.

Short-period incubations with different concentrations of Nbs^{6}ITP in the presence of Mg^{2+} ions at 37 °C and various ATP concentrations have been carried out to demonstrate the type of inhibition. Under the conditions applied, the binding of Nbs^{6}ITP to the ATPase is fully reversible as shown with radioactive Nbs^{6}ITP, which was labeled with ^{32}P in the β and γ positions. If the ATPase is incubated with [^{32}P]Nbs^{6}ITP for only a few minutes, the radioactive nucleotide can be removed completely by passing the enzyme through a Sephadex G-50 column equilibrated with Tris/HCl buffer (pH 8.0) containing 50% glycerol. The kinetic data of the short-period incubations plotted in a Lineweaver–Burk diagram revealed that Nbs^{6}ITP functions as a competitive inhibitor of F_1. If $1/v$ is plotted vs. [I], according to the method of Dixon a K_i value of 4×10^{-4} M is obtained for pH 8.0 and 1 mM Mg^{2+} (Figure 1).

Long-period incubations of the F_1 ATPase with Nbs^{6}ITP (or Dnps^{6}ITP) in Tris/HCl buffer (pH 8.0) at 37 °C yield a slow, irreversible inhibition of the catalytic activity. Mg^{2+} ions are essential for this "long-term" inhibition. Figure 2 depicts the log of the residual enzymic activity as a linear function of the time at various inhibitor concentrations. The reaction fits in best with the assumption of a mechanism according to:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}I$$

as deduced from the plot of the inactivation half-lives (τ) vs. the reciprocal inhibitor concentration (Höckel et al., 1978).

The conversion of the enzyme–nucleotide complex (EI) into the inactive "firm" nucleotide–enzyme complex (E–I) is the rate-limiting step. The K_m value for this reaction obtained from the τ vs. $1/[I]$ plot is 4×10^{-4} M. This value is, within experimental accuracy, identical with the K_i value of the competitive ("short-term") inhibition. These results indicate that the competitive inhibition of the ATPase and the irreversible "long-term" inhibition occur by binding of Nbs^{6}ITP to the same ATP binding site(s).

The quantitative evaluation of the "long-term" inhibition by [^{32}P]Nbs^{6}ITP has revealed that we have to distinguish between loosely bound, "firmly" bound (strongly adsorbed), and covalently bound nucleotides. After the incubation the modified ATPase was freed from excess and loosely bound nucleotides by passing the enzyme through a Sephadex G-50 column equilibrated with 50 mM Tris/HCl buffer (pH 8.0), which contained 50% glycerol and 4 mM EDTA. The noncovalently bound nucleotides which are not removed from the enzyme by gel filtration in glycerol-containing buffer are designated as "firmly" bound. The radioactivity of the modified ATPase after gel filtration in the glycerol buffer thus represents the number of "firmly" bound nucleotides including the covalently bound nucleotides. It must be noted here that the "tightly" bound nucleotides from mitochondrial F_1 preparations could be completely released by a similar procedure (Garrett & Penefsky, 1975). Both "firmly" bound nucleotides from *Micrococcus* sp. and "tightly" bound nucleotides from mitochondria cannot be removed by gel filtration in aqueous (Tris) buffer or ammonium sulfate precipitation. The amount of covalently bound nucleotides was then determined in the following way. After the gel filtration in the glycerol buffer the ATPase containing only "firmly" and covalently bound nucleotides was denatured in 2% sodium dodecyl sulfate and subjected to sodium dodecyl sulfate gel electrophoresis on 7.5% polyacrylamide gels. The gel pattern of the modified ATPase was the same as that of the unmodified ATPase. The noncovalently bound nucleotides migrated faster than the bromophenol blue front marker. The amount of the covalently bound nucleotides was calculated from the radioactivity incorporated into the protein bands on the gel. Figure 3 shows that there exists a linear relationship between the number of nucleotides being "firmly" plus covalently bound to the enzyme and the degree of inhibition. At 80% inhibition 4.8 nucleotides were bound so firmly to the ATPase complex that they could not be removed by gel filtration in glycerol containing media, nor by precipitation with ammonium sulfate. Of those 4.8 bound

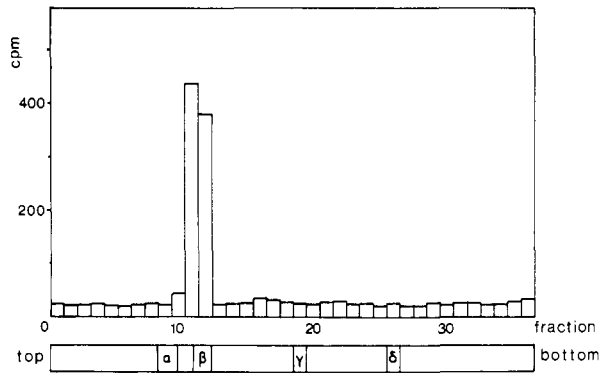

FIGURE 4: Graphical demonstration of the radioactivity distribution representing the [^{32}P]ITP nucleotides covalently attached related to the positions of the subunits of the F_1 ATPase on a sodium dodecyl sulfate-polyacrylamide gel. Note that eminent radioactivity was only found at the position of the β subunit; 20 μg of ATPase was applied to the gel; residual enzyme activity was 33%; the ϵ subunit was not detected.

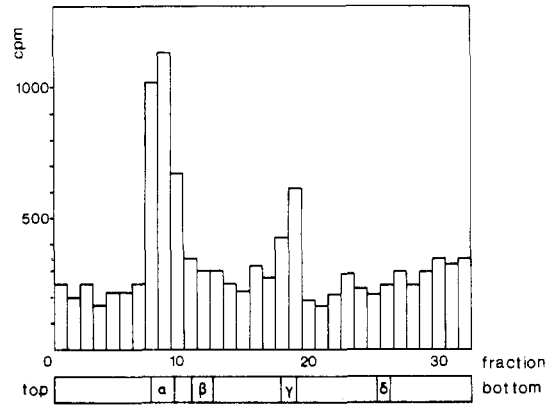

FIGURE 5: Distribution of SH groups on the subunits of F_1 ATPase labeled by reaction with 5,5'-[^{14}C]dithiobis(2-nitrobenzoic acid). ATPase was denatured by sodium dodecyl sulfate. The subunits were separated on sodium dodecyl sulfate-polyacrylamide gels. Only the α and γ subunits carry SH groups; 20 μg of ATPase was applied to the gel; the ϵ subunit was not detected.

nucleotides 1.7 were covalently bound to the enzyme.

Extrapolation to 100% inhibition could be correlated with the incorporation of a total of six "firmly" and covalently bound nucleotides per enzyme molecule. Although extrapolation of the curve of covalently bound nucleotides is not possible it can be estimated that at least two nucleotides have reacted covalently with one F_1 molecule at complete inactivation. Our calculations are based on a molecular weight of 380 000 for the ATPase as determined with the sedimentation equilibrium method. An uncertainty of 50 000 has to be taken into consideration because of the errors in the determination of the molecular weight and the possibility of losing the ϵ-polypeptide during the incubation and gel filtration procedures. Therefore and because of the error of the above analysis the number of "firmly" as well as covalently bound nucleotides per enzyme molecule should be regarded with an accuracy of about 20%. At a lower degree of inactivation, the fraction of the covalently bound nucleotides in relation to the total of all ("firmly" and covalently) bound nucleotides seems to be somewhat lower than at a higher degree of inactivation.

For identification of the subunits labeled by the ATP analogue, the gels have been sliced both in the nonstained and in the stained states. After digestion of the gel slices with solubilizer the radioactivity has been measured. Figure 4 shows the distribution of the radioactivity. The positions of the ATPase subunits are marked at the bottom. In this experiment the inhibition of the catalytic activity of the modified ATPase applied to the gel was 67%. At degrees of inhibition up to 80%, the covalently bound nucleotides became exclusively associated with the β subunit, whose molecular weight is about 55 000.

We have outlined above that the affinity label Nbs^{6}ITP preferentially reacts with aliphatic SH groups. From the results of the preceding section the presence of cysteinyl residues at the ATP binding site(s) of the β subunit should be expected. To test this inference the enzyme was incubated with 5,5'-[^{14}C]dithiobis(2-nitrobenzoic acid) (Ellman's reagent) in the presence of sodium dodecyl sulfate. Subsequently, the enzyme subunits were separated by sodium dodecyl sulfate gel electrophoresis, the gels were sliced, and the radioactivity was counted after digestion of the slices. As shown in Figure 5 the bulk of the radioactivity was found at the position of the α and γ subunits at a ratio of about 3:1, but no significant radioactivity could be detected at the position of the β subunit.

From this result it can be concluded that the affinity label Nbs^{6}ITP reacts with functional groups of the β subunit different from SH groups.

Discussion

The dissociation of the "small" subunits (γ, δ, ϵ) from the F_1 ATPase produces an enzyme complex which still possesses full enzymic activity (Höckel et al., 1976). We must therefore infer that the active sites for the ATP hydrolysis can only be located at the α and/or β subunit(s) of the enzyme. A first approach to locate the ATP binding sites of mitochondrial ATPase was made with 4-chloro-7-nitrobenzofurazan (Nbf-Cl) (Deters et al., 1975; Ferguson et al., 1975). From these data Racker (1976) surmised that an "active site" might be located on the β subunit. Ferguson et al. (1976) assumed that the modification of a tyrosine residue "inhibits the enzyme by preventing a step subsequent to a conformational change produced by addition of ATP to the enzyme". This modification did not prevent substrate binding (Ferguson et al., 1975).

The ATP analogue used in our experiments for the affinity labeling of the ATP binding sites of the plasma membrane F_1 ATPase from *Micrococcus* sp. is a nucleotide derivative which has the ability to form covalent bonds to the protein. Although Nbs^{6}ITP reacts preferentially with SH groups, this affinity label becomes exclusively bound to the β subunit of this ATPase, which did not contain any SH groups. Senior (1975) also could not find any mercapto groups on the β subunit of mitochondrial F_1 using *N*-ethylmaleimide.

The affinity label Nbs^{6}ITP is a competitive inhibitor of the F_1 ATPase from *Micrococcus* sp. with a K_i value of 0.4 mM. Furthermore, the ATP analogue induces a time-dependent irreversible inactivation of F_1. The K_m value of that reaction is identical with the K_i of the competitive inhibition within experimental accuracy. Therefore, binding to the same binding site(s) in both cases is concluded.

The time-dependent irreversible inactivation is linearly correlated with the successive binding of six ATP analogues to the enzyme molecule which could not be removed by gel filtration in glycerol containing buffer. Sodium dodecyl sulfate disc electrophoresis showed that only a part of those bound nucleotide analogues are covalently bound, e.g., at 80% inhibition 1.7 out of a total of 4.8 bound nucleotides are covalently bound to the enzyme. It is obvious that there is a discrepancy between our results and the "tight" binding of nucleotides to mitochondrial F_1 studied by Garrett & Penefsky (1975). These authors showed that all "tightly bound" nucleotides were released by gel filtration in a similar glycerol containing media. However, the methods are not completely identical. In addi-

tion, differences in the origin and preparation procedure of the ATPases may cause a different resistance of the nucleotide–enzyme complexes.

As pointed out elsewhere (Höckel et al., 1978) "long-term" irreversible inactivation of F_1 induced by Nbs^{6}ITP and other nucleotides supports an enzymic reaction mechanism involving conformational changes. The affinity labeling of F_1 by Nbs^{6}ITP described here indicates the participation of at least two nucleotide binding sites on the β subunit(s) in the catalytic process.

The labeling of regulatory or allosteric nucleotide binding sites—if they exist at all—by Nbs^{6}ITP seems to us less probable because of the following inferences. In model experiments Nbs^{6}ITP reacted exclusively with SH groups at pH 8.0 (Fasold et al., 1977). Prerequisites for the reaction with a functional residue different from an SH group are a definite steric constellation and an activation of the functional residue by the (enzymic) microenvironment. The activation of functional groups usually occurs at catalytic sites and not at regulatory/allosteric sites. However, the labeling of nucleotide sites which are not directly involved in the catalytic process cannot be totally excluded.

While this manuscript was in preparation Wagenvoord et al. (1977) reported about the photolabeling of beef-heart mitochondrial ATPase with 8-azido-ATP. The enzymic activity was completely inhibited when two molecules of 8-azido-ATP were bound per ATPase molecule. The nucleotides were also bound to the β subunit.

Acknowledgments

F.W.H. is grateful to Professor Dr. H. Fasold for facilities, support, and interest and to Miss Martina Schütrumpf for skilled technical assistance. The authors thank Dr. V. Thiele for carrying out the molecular weight determinations.

References

Abrams, A., Jensen, C., & Morris, D. (1975) *J. Supramol. Struct. 3,* 261.

Boyer, P. D. (1977) *Annu. Rev. Biochem. 46,* 957–966.

Cantley, L. C., & Hammes, G. G. (1975) *Biochemistry 14,* 2968.

Clayton, S. J., & Fitzgibbon, W. E. (1968) *Synth. Proced. Nucleic Acid Chem. 1,* 279.

Deters, D. W., Racker, E., Nelson, N., & Nelson, H. (1975) *J. Biol. Chem. 250,* 1041.

Fasold, H., Hulla, F. W., Ortanderl, F., & Rack, M. (1977) *Methods Enzymol. 46,* 289.

Faust, U., Fasold, H., & Ortanderl, F. (1974) *Eur. J. Biochem. 43,* 273.

Ferguson, S. J., Lloyd, W. J., Lyons, M. H., & Radda, G. K. (1975) *Eur. J. Biochem. 54,* 117.

Ferguson, S. J., Lloyd, W. J., Radda, G. K., & Slater, E. C. (1976) *Biochim. Biophys. Acta 430,* 189.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66,* 375.

Garrett, N. E., & Penefsky, H. S. (1975) *J. Biol. Chem. 250,* 6640.

Hampton, A., & Maguire, M. H. (1961) *J. Am. Chem. Soc. 83,* 150.

Höckel, M., Hulla, F. W., Risi, S., & Dose, K. (1976) *Biochim. Biophys. Acta 429,* 1020.

Höckel, M., Hulla, F. W., Risi, S., & Dose, K. (1978) *J. Biol. Chem.* (in press).

Hulla, F. W., & Fasold, H. (1972) *Biochemistry 11,* 1056.

Hulla, F. W., Höckel, M., Risi, S., & Dose, K. (1976) *Eur. J. Biochem. 67,* 469.

Lee, S. H., Kalra, V. K., Ritz, C. J., & Brodie, A. F. (1977) *J. Biol. Chem. 252,* 1084.

Leimgruber, R. M., & Senior, A. E. (1976) *J. Biol. Chem. 251,* 7103.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

Maeda, M., Kobayashi, H., & Futai, M. (1976) *Biochem. Biophys. Res. Commun. 70,* 228.

Michelson, A. M. (1964) *Biochim. Biophys. Acta 91,* 1.

Moffat, I. G. (1964) *Can. J. Chem. 42,* 599.

Oppenheim, J. D., & Salton, M. R. J. (1973) *Biochim. Biophys. Acta 298,* 297.

Pederson, P. L. (1975) *Biochem. Biophys. Res. Commun. 64,* 610.

Racker, E. (November, 1976) *Trends Biochem. Sci.,* 244.

Risi, S., Höckel, M., Hulla, F. W., & Dose, K. (1977) *Eur. J. Biochem. 81,* 103–109.

Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250,* 7848.

Senior, A. E. (1975) *Biochemistry 14,* 660.

Slater, E. C. (1974) *Biochem. Soc. Trans. 2,* 1149.

Tsuchiya, T., & Rosen, B. P. (1975) *Biochem. Biophys. Res. Commun. 63,* 832.

Wagenvoord, R. J., van der Kran, I., & Kemp, A. (1977) *Biochim. Biophys. Acta 460,* 17.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244,* 4406.

Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50,* 5065.